Colorimetric Quantification of α-D-galactose 1-phosphate

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Summary

Colorimetric quantification of α-D-galactose 1-phosphate (Gal 1-P) was performed by adding UDP-glucose hexose-1-phosphate uridylyltransferase and UDP-D-glucose to the conventional α-D-glucose 1-phosphate assay using phosphoglucomutase and glucose 6-phosphate dehydrogenase. This method is very specific to Gal 1-P and results in a linear calibration curve. The method was applied to continuous monitoring of the lacto-*N*-biose phosphorylase reaction. By adding mutarotase, galactokinase, and ATP, the method can be utilized for the quantification of D-galactose.

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α-D-Galactose 1-phosphate (Gal 1-P) is a key compound in D-galactose metabolism via the Leloir pathway. This pathway is characterized by the successive actions of the enzymes, aldose 1-epimerase (GalM), galactokinase (GalK), UDP-glucose hexose-1-phosphate uridylyltransferase (GalT) and UDP-glucose 4-epimerase (GalE), all of which are involved in the conversion of galactose to α-D-glucose 1-phosphate (Glc 1-P) (de Vos and Vaughan, 1994). Recently, we reported a novel galactose metabolic pathway in *Bifidobacterium longum*, in which the lacto-*N*-biose phosphorylase (LNBP, 1,3-β-D-galactosyl *N*-acetylhexosamine phosphorylase [EC 2.4.1.211]) gene was found in the galactose metabolism gene cluster (Kitaoka *et al*., 2005). In this pathway, the key enzyme is LNBP, which reversibly phosphorolyzes 1,3-β-D-galactosyl *N*-acetylhexosamine to Gal 1-P and *N*-acetylhexosamine (Derensy-Dron *et al*., 1999). This pathway does not require a galactokinase, unlike the Leloir pathway, because LNBP directly produces Gal 1-P from 1,3-β-galactosyl *N*-acetylhexosamine. Since 1,3-β-D-galactosyl *N*-acetylhexosamine units are the core structures of mucin sugars (galacto-*N*-biose, 1,3-β-D-galactosyl *N*-acetyl-D-galactosamine) and human milk oligosaccharides (lacto-*N*-biose I, 1,3-β-D-galactosyl *N*-acetyl-D-glucosamine) (Hoskins *et al*., 1985; Kunz *et al*., 2000), this pathway may be important especially for the preferential proliferation of bifidobacteria in the intestine of human newborns.

We recently reported a kilogram-scale production of lacto-*N*-biose I using the phosphorylase (Nishimoto and Kitaoka, 2007), indicating the possible utilization of phosphorylases to produce galactosides. Since LNBP is the only phosphorylase known until date that degrades galactosides (Kitaoka and Hayashi, 2002), it is valuable to find a novel phosphorylase specific to different galactosides. During the process to find such enzymes by conventional screening or protein engineering, detection, and quantification of Gal 1-P on a large number of samples are required.

Although Gal 1-P can be quantitatively measured using chromatographic techniques such as HPLC, these assays are often cumbersome and time consuming and can only process a limited number of samples. Quantification of sugar phosphate is possible using mass spectrometry (Zea and Pohl, 2004; Zea and Pohl, 2004), but it requires expensive equipments. Thus, alternative colorimetric methods for quantifying Gal 1-P are highly desirable, which can process a large number of samples accurately. Gal 1-P can be quantitatively detected by a galactose oxidase or an NAD^+-dependent galactose dehydrogenase reaction after being converted to D-galactose by an alkaline phosphatase (Avigad *et al*., 1962; Diepenbrock *et al*., 1992). However, these conventional methods cannot quantify Gal 1-P in the presence of D-galactose.

In this paper, we describe a simple colorimetric method for quantifying Gal 1-P. This method involves the addition of UDP-D-glucose (UDP-Glc) and GalT to the reagents of a Glc 1-P assay, which contains NAD^+, phosphoglucomutase (PGM), and glucose 6-phosphate dehydrogenase (G6PDH) (Michael, 1984) (Figure 1). In this method, Gal 1-P and UDP-Glc are converted into UDP-Gal and Glc 1-P, respectively, by GalT, and the resultant Glc 1-P is ultimately converted by PGM and G6PDH into NADH, which can be quantified by its absorbance at 340 nm. NADH was detected at a visible wavelength by substituting thio-NAD^+ for NAD^+. LNBP

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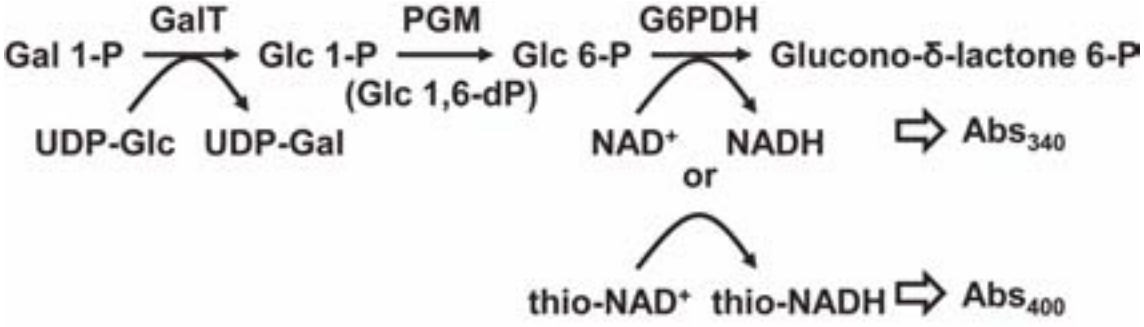

Fig.1. Schematic diagram for the colorimetric quantification of Gal 1-P.

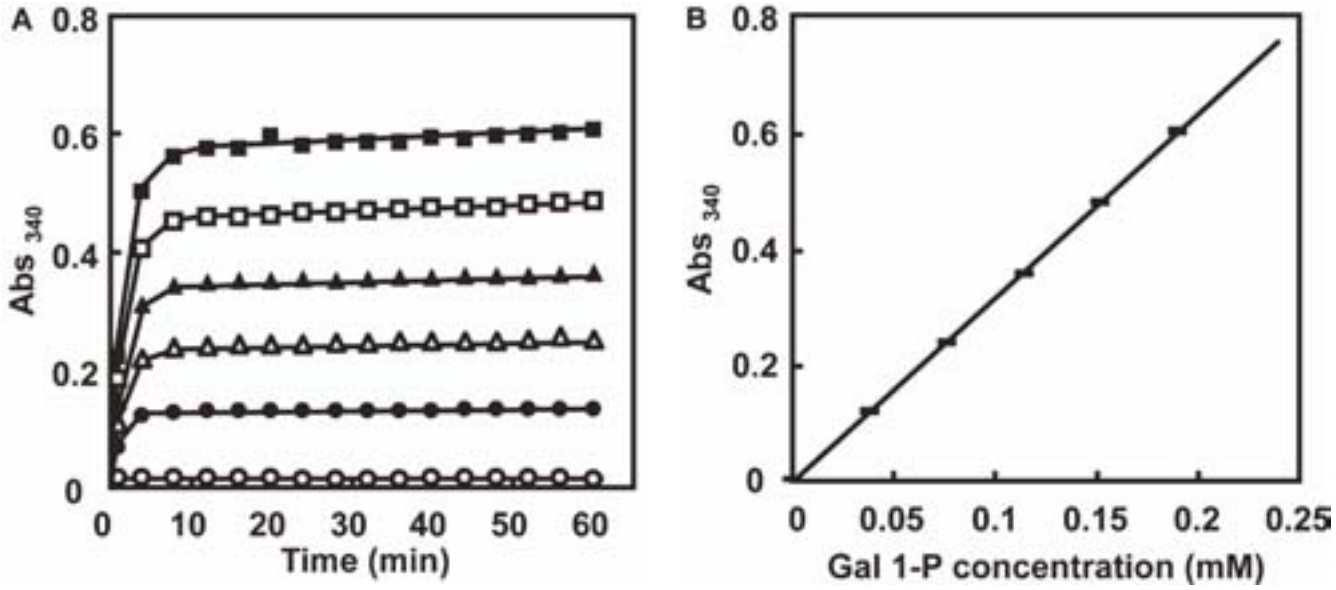

Fig.2. Time course and calibration curve for the colorimetric quantification of Gal 1-P using NAD^+. (A) Time course of color development during the quantification of Gal 1-P using NAD^+. Gal 1-P concentrations were 0 mM (open circles), 0.038 mM (closed circles), 0.076 mM (open triangles), 0.114 mM (closed triangles), 0.152 mM (open squares), and 0.190 mM (closed squares). (B) Calibration curve of Gal 1-P using NAD^+. The measurements were performed in triplicate and the standard deviations are given in error bars.

activity was evaluated by using the Gal 1-P assay. In addition, we applied this method to the quantification of Gal by adding some components to the reagents used in the Gal 1-P assay.

Recombinant LNBP (Kitaoka *et al*., 2005) and GalT (Nishimoto and Kitaoka, 2007.) from *B. longum* JCM1217 with additional His_6-tag sequences at their C-terminal were prepared as described. It should be noted that GalT from yeast is commercially available from Sigma (Catalog number G4356: St. Louis, MO). The *galK* gene from *B. longum* JCM1217 (GenBank accession number AB303573) was amplified by PCR using the following primers containing NdeI or XhoI restriction endonuclease sites (indicated by underlining) for ligation to the expression vector: Forward, GATATACATATGACTGCTGTTGAATTCATTGAGC; reverse GATTTACTCGAGTGCCTCGCGTGAAGCGGATTT CGCCGC. The amplified fragment was inserted into pET-30 (Novagen Inc., Madison, WI) at the NdeI and XhoI sites using a Ligation High DNA ligation kit (Toyobo, Osaka, Japan) to

generate the expression vector. Recombinant GalK with His6-tag sequences at its C-termini was produced by Escherichia coli BL21 (DE3) cells carrying the vector and purified by Ni-NTA Agarose (QIAGEN, Hilden, Germany) column chromatography, using methods identical to those for LNBP and GalT (Kitaoka *et al.*, 2005; Nishimoto and Kitaoka, 2007). The specific activity of GalK was 174 U/mg-protein. GalK is also commercially available from GenWay (Catalog number: 10-288-22113F: San Diego, CA). Mutarotase from the kidney of pig, PGM from rabbit muscle, G6PDH from Leuconostoc mesenteroides, ATP and NAD^+, and thio-NAD^+ were obtained from Oriental Yeast Co. Ltd (Tokyo, Japan). Gal 1-P and α-D-glucose 1,6-bisphosphate (Glc 1,6-dP) were purchased from Sigma, and D-galactose and UDP-Glc were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals used in this study were reagent grade.

Working reagent A for the Gal 1-P assay was prepared by mixing 0.5 IU/ml GalT, 2.5 IU/ml PGM, 2 IU/ml G6PDH, 20 μM Glc 1,6-dP, 2 mM NAD^+, and 2 mM UDP-Glc in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. An aliquot (100 μl) of solution containing 0-0.25 mM Gal 1-P was mixed with 50 μl of working reagent A and 50 μl of working reagent B (50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$) and incubated at 37 ℃ for 30 min. Gal 1-P concentration was measured by determining absorbance at 340 nm, without stopping the reaction. When thio-NAD^+ was substituted for NAD^+, absorbance was measured at 400 nm.

The time course of Gal 1-P generated using the above protocol was monitored at 37 ℃ (Figure 2A). The reaction was complete after 20 min and absorbance did not decrease significantly thereafter, up to 60 min. We therefore selected a reaction time of 30 min for this protocol. When we measured the relationship between Gal 1-P concentration and Abs_{340} using the above protocol, with triplicate measurements at each Gal 1-P concentration, we found that the Gal 1-P calibration curve was linear in the concentration range from 0 to 0.25 mM (Figure 2B). The following relationship was derived from linear regression, with a correlation coefficient greater than 0.999:

$y = 3.19x - 0.0017$ (x = [Gal 1-P]/mM; $y = Abs_{340}$).

When NAD^+ was converted to thio-NAD^+ and absorbance was measured at 400 nm, the Gal 1-P calibration curve was linear in the concentration range from 0 to 0.08 mM, and the following relationship was derived from regression, with a correlation coefficient greater than 0.998:

$y = 6.21x + 0.0019$ (x = [Gal 1-P]/mM; $y = Abs_{400}$).

Because the molar absorption coefficient of thio-NADH at 398 nm was nearly twice that of NADH at 340 nm (Karrer and Schukri, 1945), the protocol using thio-NAD^+ could measure the quantity of Gal 1-P with greater sensitivity than the protocol using NAD^+. Moreover, measurements of Gal 1-P were not altered by the presence of galactose, methyl-β-D-galactoside, melibiose, L-arabinose, *N*-acetyl-D-galactosamine, or D-galactosamine.

Continuous monitoring of Gal 1-P produced by LNBP was examined. Lacto-*N*-biose I (final concentration 10 mM), sodium phosphate buffer (pH 7.5, final concentration 10 mM), and LNBP at various concentrations were substituted for Gal 1-P in the assay mixture with thio-NAD^+, and the mixtures were incubated at 30℃. The absorbance at 400 nm linearly increased with reaction time because of the phosphorolysis of the lacto-*N*-biose. Moreover, when enzyme concentration increased, the slope of the reaction increased linearly, indicating that the rate determining step of this assay system was that involving LNBP, not the conversion of Gal 1-P to thio-NADH, making LNBP activity continuously measurable without a stop reaction.

The Gal 1-P assay method was also applied to the measurement of D-galactose by adding several components. To detect galactose, working reagent B was replaced by working reagent C, consisting of 2 IU/ml mutarotase, 4 IU/ml GalK and 2 mM ATP in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The calibration curve for D-galactose was also linear in the concentration range from 0 to 0.1 mM, and the correlation coefficient for regression was greater than 0.998 (data not shown). The D-galactose assay was not influenced by the presence of galactose, methyl-β-D-galactoside, melibiose, L-arabinose, or *N*-acetyl-D-galactosamine, but was altered by the presence of D-galactosamine, which had a relative sensitivity of 7%. This method, however, was considerably more specific than conventional methods utilizing galactose oxidase or galactose dehydrogenase, which are sensitive to the presence of several saccharides (Avigad *et al.*, 1962; Blachnitzky *et al.*, 1974).

In conclusion, we have developed protocols for the specific colorimetric quantification of Gal 1-P by adding GalT and UDP-Glc to the conventional Glc 1-P assay using PGM and G6PDH. This method is very specific for Gal 1-P and results in a linear calibration curve. Evaluation of LNBP activity by continuous monitoring strongly indicates that this Gal 1-P colorimetric assay is both valid and accurate. In addition, we also show that galactose can be quantified by adding ATP, mutarotase, and GalK to the Gal 1-P assay system.

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α-D- ガラクトース1- リン酸の比色定量法

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要　約

α -D- ガラクトース1- リン酸（Gal 1-P）の比色定量として、ガラクトース定量法にホスファターゼを組み合わせる方法が知られるが、ガラクトースおよびガラクトース含有オリゴ糖が混在する試料中での Gal 1-P の定量法としては不都合があった。そこで我々はガラクトース代謝系酵素群に着目し、Gal 1-P のみを検出および Gal 1-P とガラクトースを同時に検出する2つの比色定量法を開発した。各種濃度の Gal 1-P に発色試薬（UDP- グルコース - ヘキソース -1- リン酸ウリジリルトランスフェラーゼ、ホスホグルコムターゼ、グルコース -6- リン酸デヒドロゲナーゼ、NAD^+ または Thio-NAD^+、UDP- グルコース、グルコース1,6- ビスリン酸、$MgCl_2$含有トリス緩衝液）を混合し、経時的に340 nm（NAD^+）または400 nm（Thio-NAD^+）の吸収を測定した結果、20分程度で平衡に達し吸光度は Gal 1-P 濃度に比例した。また上記試薬にムタロターゼ、ガラクトキナーゼおよび ATP を追加することにより、各種濃度のガラクトースに対し同様の結果が得られた。また加リン酸分解酵素ラクト -*N*- ビオースホスホリラーゼの反応によって生じる Gal 1-P を、発色試薬中にて反応停止操作を伴なわず連続的にモニタリングした結果、算出された活性値は各酵素濃度に比例しており、本定量法を用いた活性測定法の妥当性・有用性が示された。

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